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Co-suppression in transgenic *Petunia hybrida* expressing chalcone synthase A (*chsA*)

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Abstract Chalcone synthase A is a key enzyme in the anthocyanin biosynthesis pathway. Expression of *chsA* gene in transgenic *Petunia hybrida* resulted in flower color alterations and co-suppression of transgenes and endogenous genes. We fused the -glucuronidase (*uidA*) gene to the C-terminal of *chsA* gene, and transferred the fusion gene into *Petunia hybrida* via *Agrobacterium tumefaciens.* GUS histochemical staining analysis showed that co-suppression occurred specifically during the development of flowers and co-suppression required the mutual interaction of endogenous genes and transgenes. RNA *in situ* hybridization analysis suggested that co-suppression occurred in the entire plant, and RNA degradation occurred in the cytoplasm.

Keywords: chalcone synthase A, co-suppression, *Petunia hybrida* , *in situ* hybridization.

Over the last decade, a large number of transgenic plants have been generated to modify or improve different traits of crops. Unexpectedly, in some cases when the transgene is homologous to an endogenous gene, the expression of both the transgene and the endogenous gene may become co-suppressed, which is called the co-suppression phenomenon [1,2] . Co-suppression has been observed in many kinds of plants, even in bacteria, fungi and mammalian systems [3] . The occurrence of co-suppression has caused failures of many cases of genetic engineering, but the mecha-

nism of co-suppression may reveal some very interesting aspects of gene regulation. Therefore, it draws the interest of many biologists. Nuclear run-on assays indicated that co-suppression occurred after the transcription of the transgene, and it was then called post-transcriptional gene silencing, PTGS [4,5]. Some hypotheses were proposed for the mechanisms of co-suppression, such as the threshold and RNA-dependent RNA polymerase (RdRp) hypothesis [6,7].

Introduction of genes associated with pigment synthesis into *Petunia hybrida* can lead to co-suppression, as is seen in the case of *chsA* gene. Chalcone synthase is a key enzyme in the pathway of anthocyanin biosynthesis. Suppression of chalcone synthase expression in transgenic *Petunia hybrida* transformed with sense or anti-sense *chsA* transgenes therefore leads to losses of anthocyanin pigment on petals, and the flower alters from purple to white or purple with white sectors [1,2,8]. Due to the visibility of the flower pigmentation, the transgenic petunia becomes a

nice model system for co-suppression study. *ChsA* gene is transcriptionally activated in the epidermal cells of flower petals during the flower development in wild petunia [9–11]. When co-suppression of *chsA* occurs in transgenic petunia, will it be limited to certain development period of flowers or will it also occur in other tissues of other periods of the development? To address these questions, we fused the -glucuronidase (*uidA*) gene to the C-terminal of *chsA* gene, cloned into a plant expression vector with a CaMV 35S promoter, and transferred the chimerical *chsA-uidA* gene into *Petunia hybrida* via *Agrobacterium tumefaciens.* By GUS histochemical localization and RNA *in situ* hybridization, we studied the co-suppression in different periods of the development.

1 Materials and methods

1.1 Materials and reagents

E. coli strain DH5 and *Agrobacterium tumefaciens* strain LBA4404 were stored in our lab. Plasmid pBI121 was product of Clontech. *Petunia hybrida* "pure pink" was one of our collections.

DIG RNA labeling kit, anti-Dig AP were the products of Roche. Other chemicals were Sigma products or analytically pure reagents made in China.

1.2 Construction of expression plasmid

The complete *chsA* coding sequence was amplified by PCR with two primers of CHS *Xba* I (5 -CC TCT AGA AAA ATG GTG ACA GTC GAG GAG TAT CGT-3) to introduce a *Xba* I site upstream of the translation start codon and CHS *BamH* I (5 -AC GGA TCC AGC AAC ACT GTG GAG GAC AAC AGT-3) to delete the termination codon and introduce a *BamH* I enzyme diges-

tion site. Plasmid pBI121-chsA was constructed by inserting the *Xba* I and *BamH* I digested *chsA* PCR fragment downstream of the CaMV 35S promoter and fused with *uidA* gene in the same open reading frame. Plasmid pBI121 was used as a control (fig. 1).

Fig. 1. Structures of plasmid pBI121 and pBI121-chsA. nos-pro, Nopaline synthase promoter; *Npt* II, neomycin phosphotransferase; nos-ter, nopaline synthase terminater; CaMV 35S Pro, CaMV 35S promoter; *chsA*, chalcone synthase A gene; *uidA*, -glucuronidase gene.

1.3 Plant transformation

The T-DNA of pBI121-chsA was introduced into *Petunia hybrida* by *Agrobacterium tumefaciens*-mediated transformation of leaf discs. Leaves were dissected into discs of about 1 cm in

diameter, and immersed in Agrobacterium for 5 min, then transferred to an MS agar plate supplemented with BA 1 mg/L, IAA 0.01 mg/L. Transformants were selected by 100 mg/L kanamycin, new seedling differentiated. Then media changed to MS media with antibiotic but no hormone.

1.4 Southern blot analyses

Total DNA was prepared from leaves using the method of hexadecyltrimethylammonium bromide (CTAB). About 0.5 to 1.0 g leaves were ground in liquid nitrogen, and then 500 ∝L CTAB buffer (2% CTAB, 1.4 mol/L NaCl, 0.2% mercaptoethanol, 20 mmol/L EDTA pH8.0, 100 mmol/L Tris g HCl pH8.0) was added and mixed with the liquid. After incubated in water bath at 65 for 30 min, DNA was extracted by equal volume of chloroform: isoamyl alcohol (24 1). The mixture was centrifuged at 5000 h *g*/min for 3 min. The supernatant was transferred into a new tube and equal volume isopropanol was added. The genomic DNA was centrifuged at 10000 h *g*/min for 10 min and the pellet was washed with 70% ethanol and air dried. The DNA pellet was resuspended in TE and stored at −20 . 10 ∝g plant DNA samples were digested with restriction enzymes, electrophoresed in a 0.8% agarose gel, and blotted according to Sambrook et al.[12] To avoid the disturbance of endogenous *chsA*, *uidA* and *Npt* II were used as the probes. Radioac-

tively labeled probes were generated by the random prime method.

1.5 Northern blot analyses

Total RNA was extracted from leaves and flower buds using the method of guanidine thiocyanate-phenyl-chloroform. Northern blot was carried out according to Sambrook et al.[12] DIG-11-dUTP labeled *chsA* cDNA fragment and DIG-11-dUTP labeled *uidA* cDNA fragment were used as probes. Hybridization temperature was 68 .

1.6 GUS histochemical staining

GUS histochemical staining was performed according to Jefferson et al.[13] Fresh plant materials were incubated in the staining solution at 37 for 2 ü 30 h, bleached in 70% alcohol until the materials became white. The staining solution contained 100 mmol/L $NaPO_4$, pH 7.0; 10 mmol/L EDTA pH 8.0; 0.1% Triton X-100; 1.0 mmol/L X-Gluc, stored at −20 .

1.7 RNA *in situ* hybridization

RNA *in situ* hybridization was carried out according to Cox et al.[14] Paraffin embedded tissues were cut into 10 ∝m sections. After being baked at 180 over night, slides were treated with poly-l-lysine (0.1%, *m* /*v*).

DIG-labeled RNA probes were performed following the protocol of Roche RNA labeling kit. *ChsA* gene was cloned in vector pGEM(7zf+) and digested either at the 5 or 3 end to obtain the sense *chsA* RNA or the antisense *chsA* RNA.

The sections were dewaxed by xylenes and hydrated in a series of diluted ethanol (from 100% ethanol to H_2O). After digested by proteinase K at 37 for 30 min, the sections were de-

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hydrated and hybridized at 52 over night. Then the sections were washed twice at 52 for 1 h each time. An anti-DIG-AP was used in the immunological detection and color reaction was carried out with NBT/BCIP over night. The slides were mounted and photographed under a microscope.

Hybridization solutions: (1) 10 h hybridization salts 3 mol/L NaCl, 0.1 mol/L Tris-HCl (pH6.8), 0.1 mol/L PBS, 50 mmol/L EDTA in DEPC-treated water; (2) hybridization buffer: 50% distilled formamide 500 ∝L, 10 h hybridization salts 100 ∝L, 50% dextran sulfate 250 ∝L, 100 mg/mL Yeast tRNA 12.5 ∝L, DEPC-treated water 185 ∝L per mL. Wash solution: 2 h SSPE.

2 Results

2.1 Generating transgenic plants and Southern blotting analysis

To study the co-suppression in transgenic petunia, we constructed a plant expression plasmid carrying a fused *chsA* and *uidA* gene (fig. 1). The fused *chsA* and *uidA* gene was transferred into *Petunia hybrida* via *Agrobacterium tumefaciens* -mediated transformation of leaf discs. Co-suppression occurred in about 20 transgenic petunia plant and the flower was changed from purple to white or white and purple. For the Southern blotting analysis of the transgenic petunia plant, *uidA* and *Npt* II were used as the probes to avoid the disturbance of endogenous *chsA* gene. Genome DNA was digested by *Eco* R I and *Hin* d III or both of them. Hybridization results showed that the target genes were integrated into the genome of transformed petunia plants in more than one copy (fig. 2).

Fig. 2. Southern blot analysis of DNA from transgenic petunia. Southern blot analysis of samples CG1 and CG3 are presented. Genomic DNA was digested with *Eco* R I (E), *Hin* d III (H) or both of them (E,H). *UidA* and *Npt* II were used as probes. /*Hin* d III was used as DNA marker.

2.2 Northern blotting analysis of transgenic plants

The total RNA was extracted from leaves and flower buds (2 0 3 cm) of the untransformed petunia, transgenic petunia plant and the white part and purple part of fully opened flowers of *chsA* -*uidA* transgenic petunia plant. DIG-11-dUTP labeled *chsA* cDNA fragment was used as probe to perform Northern blotting (fig. 3). In lines 3 and 4, there were weak hybridization signals.

It showed that co-suppression had started and *chsA* gene had a very low expression. Therefore the corollas of the flowers showed a type of white in purple. Neither the white part nor the purple part of the fully opened flowers of transgenic petunia showed any hybridization signal. We also did another Northern blotting using samples of total RNA extracted from leaves of untransformed petunia plant, leaves of *chsA* -*uidA* transformed petunia plant (before flowering) and leaves of *uidA* transformed petunia plant (as control) to confirm the result. DIG-11-dUTP labeled *uidA* cDNA fragment was used as probe (lines 8, 9,10 in fig. 3). Line 10 detected *uidA* gene, and line 9 detected *chsA* -*uidA* fused gene.

Fig. 3. Northern blot analysis of transgenic petunia. Line 1 was total RNA extracted from flower buds of the untransformed petunia. Line 2 was total RNA extracted from leaves of the untransformed petunia. Line 3 was total RNA extracted from flower buds of transgenic petunia. Line 4 was total RNA extracted from leaves of transgenic petunia. Line 5 was total RNA extracted from the white part of the flowers of transgenic petunia. Line 6 was total RNA extracted from the purple part of the flowers of transgenic petunia. Line 7 was total RNA extracted from leaves of transgenic petunia (after flower fully opened). Line 8 was total RNA extracted from leaves of the untransformed petunia. Line 9 was total RNA extracted from leaves of *chsA-uidA* transformed petunia (before flowering), and line 10 was total RNA extracted from leaves of *uidA* transformed petunia (as control). 28S rRNA was used as control of loading quantity.

2.3 Time course of co-suppression in transgenic plants

Because *chsA* and *uidA* genes were fused together, we could detect the expression of *chsA* by detection of the expression of *uidA* gene. Different organs (root, leaf, stem) of transgenic petunia were histostained by X-Gluc before flowering. All of them could be dyed into blue before flowering (Plate I-1 ü 3). In addition, different lines showed different degrees of blue. According to

Koes's method of dividing flower developing stage [9], we histostained different stages of the petunia flower (Plate I-5). At the developing early stage of the flower, sepal, stamen, little corolla could be dyed into blue. With the development of flower, the color became paled to colorless. At the same time the other organs could not be dyed either.

2.4 Localization of co-suppression by RNA *in situ* hybridization

The different organs of flowered transgenic and wild petunia were sampled and prepared for paraffin slides and hybridized by using DIG labeled *in vitro* transcripts of *chsA* gene. As shown in Plate II, 1, 4, 7, 9 were the hybridization results of leaf, stem, root, corolla of the transgenic petunia, and signals could be detected in all the cells of both the nucleus and cytoplasm using *chsA* antisense transcripts as probe. But no signal could be detected using sense *chsA* RNA as the probe (Plate II-2, 5, 8 and 10). While in wild petunia, signals could only be detected in inner and upper epidermal cells of corolla using *chsA* antisense transcripts probe (Plate II-3, 6, 11, 12).

3 Discussion

Biologists have made great efforts to study the mechanism of co-suppression in recent years. The results showed that the copy number, DNA methylation and structure of the integrated T-DNA of the transgene may play a role in the process of co-suppression [6,7]. The results also showed that RNA-dependent RNA polymerase may be involved in the RNA degradation [15,16]. In the study of signal transduction, small signal molecules such as small RNA molecules were detected [17]. In this research, we address this question from the aspects of localization of RNA degradation and co-suppression regarding plant development.

In this research, a fuse gene including *chsA* and *uidA* was transferred into petunia. Compared with wild petunia, the flower color of transformed petunia changed. Southern blotting showed that the T-DNA in transformed petunia was all multi-copy, and some inserting type was in a version of reverse repeat. Jorgensen et al.'s research revealed similar result, but they got both single and multi-copy intergrations, and the co-suppression rate was 25% [18]. In this research the co-suppression rate was 100%. We suggested that in all transgenic petunia we obtained multi-copy insertion, especially reverse repeat may play an important role in the occurrence of co-suppression.

In GUS histochemical localization experiments, the results showed that no co-suppression happened before flowering, while no GUS staining in the plant after flowering. During the period of flowering, the GUS staining became gradually pale. This may suggest that co-suppression happened during the flowering, which is consistent with the expression of endogenous *chsA* in the flower development. The results also indicated that the occurrence of co-suppression needs the mutual interaction of endogenous and transgenic gene *chsA*, this interaction is not in the level of DNA-DNA, and it needs the transcription activity of endogenous *chsA*. When co-suppression happened, no GUS staining was observed in the whole plant, which indicated that co-suppression happened in the whole plant and was not restricted to the inner and upper epidemal layers of co-

rolla in which endogenous *chsA* gene was expressed specifically. When co-suppression occurred in flower during the development, co-suppression took place in other tissues too. These results suggested that there was a signal which might be transported from flower to other part of the plant. Voinnet et al. found that there was a small RNA signal molecule with a size of 25nt involved in GFP transformed and silenced tobacco [19,20]. Hamilton detected 21 ū 25 nt small RNA molecule in gene silenced plants [17]. Hammond detected small RNA molecules in drosophila [21]. In our *chsA* transformed petunia, the small RNA signal molecules may also be involved.

In situ hybridization with RNA showed that there was no tissue specificity in co-suppression. And it also showed that no signal could be detected with the sense *chsA* RNA transcripts as a probe, which indicated that there was no free antisense *chsA* RNA in the silenced cells. In the research of Zamore, there were mRNA and dsRNA when RNA was degraded, but dsRNA was in a complex together with RNA helicase [22,23]. Our results showed that there was mRNA. More work needed to be carried out in detecting if there is dsRNA involved. Our results also showed that RNA was degraded in cytoplasm, because signals could be detected in both nucleus and cytoplasm. This result was consistent with the research on RNA-dependent virus resistance [24].

Taken together, our results revealed the development feature of co-suppression and the localization of co-suppression. The process of co-suppression in *chsA* transgenic petunia may be assumed to be like the following process. In transgenic petunis plant, *chsA* transgene was transferred and inserted into petunia genome. When endogenous *chsA* gene began to transcribe, some aberrant RNA may be produced owing to the action of repeat segment of insertion. The aberrant RNA or part of it may act as signal molecules or the template of RdRp (belonging to plant [15,16]) to synthesize complement RNA (cRNA). The cRNA may be then annealed with mRNA, and was degraded by double-stranded RNA specific RNase. The degradation of RNA would occur in cytoplasm. Further experiments deep into the process are needed.

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